Rapid Extraction of Genomic DNA from Medically Important Yeasts and Filamentous Fungi by High-Speed Cell Disruption

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Current methods of DNA extraction from different fungal pathogens are often time-consuming and require the use of toxic chemicals. DNA isolation from some fungal organisms is difficult due to cell walls or capsules that are not readily susceptible to lysis. We therefore investigated a new and rapid DNA isolation method using high-speed cell disruption (HSCD) incorporating chaotropic reagents and lysing matrices in comparison to standard phenol-chloroform (PC) extraction protocols for isolation of DNA from three medically important yeasts (Candida albicans, Cryptococcus neoformans, and Trichosporon beigeli) and two filamentous fungi (Aspergillus fumigatus and Fusarium solani). Additional extractions by HSCD were performed on Saccharomyces cerevisiae, Pseudallescheriaboydii, and Rhizopus arthrits. Two different inocula (10^8 and 10^7 CFU) were compared for optimization of obtained yields. The entire extraction procedure was performed on as many as 12 samples within 1 h compared to 6 h for PC extraction. In comparison to the PC procedure, HSCD DNA extraction demonstrated significantly greater yields for 10^8 CFU of C. albicans, T. beigeli, A. fumigatus, and F. solani (P ≤ 0.005), 10^7 CFU of C. neoformans (P ≤ 0.05), and 10^6 CFU of A. fumigatus (P ≤ 0.01). Yields were within the same range for 10^8 CFU of C. neoformans and 10^7 CFU of C. albicans for both HSCD extraction and PC extraction. For 10^7 CFU of T. beigeli, PC extraction resulted in a greater yield than did HSCD (P ≤ 0.05). Yields obtained from 10^8 and 10^7 CFU were significantly greater for filamentous fungi than for yeasts by the HSCD extraction procedure (P < 0.0001). By the PC extraction procedure, differences were not significant. For all eight organisms, the rapid extraction procedure resulted in good yield, integrity, and quality of DNA as demonstrated by restriction fragment length polymorphism (RFLP), PCR, and random amplified polymorphic DNA (RAPD) analysis.

We conclude that mechanical disruption of fungal cells by HSCD is a safe, rapid, and efficient procedure for extracting genomic DNA from medically important yeasts and especially from filamentous fungi.

The rapid availability of genomic DNA from medically important fungi is becoming increasingly important in reference clinical microbiology laboratories for accurate molecular epidemiologic subtyping and diagnostic PCR. Direct sequencing systems which will rely upon the rapid availability of genomic DNA are currently under development for clinical microbiological laboratories. Previously described methods for genomic DNA extraction from fungal pathogens require hours to days to complete and often incorporate toxic chemicals. Additionally, the release of DNA is often poor due to cell walls or capsules that are not readily susceptible to lysis.

High-speed cell disruption (HSCD) is a method of DNA extraction which permits rapid lysis of cells and recovery of nucleic acids (11). To our knowledge, such rapid extraction procedures have not been applied to medically important fungi. We therefore investigated a new rapid DNA isolation method using HSCD and incorporating chaotropic reagents and lysing matrices in comparison to standard phenol-chloroform (PC) extraction protocols for DNA isolation from three medically important yeasts (Candida albicans, Cryptococcus neoformans, and Trichosporon beigeli) and two filamentous fungi (Aspergillus fumigatus and Fusarium solani). Additional extractions were performed on Saccharomyces cerevisiae, Pseudallescheria boydii, and Rhizopus arthrits. Two different inocula (10^8 and 10^7 CFU) were compared for optimization of obtained yields. The integrity and quality of the extracted genomic DNA were further validated by restriction fragment length polymorphism (RFLP), PCR, and random amplified polymorphic DNA (RAPD) analysis.

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MATERIALS AND METHODS

Rapid extraction procedure. (i) Culture and harvesting of fungal strains. Forty-five milliliters of 2% Sabouraud medium was inoculated with one to three colonies of either C. albicans 4740, S. cerevisiae 4740 or 5645, or T. beigeli TSAS-1. C. neoformans 9658 or 134476 was inoculated into 45 ml of yeast nitrogen broth plus 2% glucose, 20 μg of uracil per ml, and 20 μg of adenine per ml. Cultures of C. albicans, S. cerevisiae, and T. beigeli were grown for 18 h at 37°C in a reciprocal shaking water bath set at 80 oscillations/min. Cultures of C. neoformans were grown for 2 to 3 days at 27°C in a reciprocal shaking water bath set at 80 oscillations/min. The cultures were harvested by centrifugation for 10 min at 2,000 × g (Damon/IEC CRU-5000 centrifuge). The cells were washed with normal saline, repelleted, and resuspended in 10 ml of normal saline.

A. fumigatus 972 and 4215, F. solani 92-1484 and 93-1547, P. boydii 91-1216, and R. arthrits 88-390 were inoculated onto potato dextrose agar slants, incubated for 24 h at 30°C, and then placed at room temperature until mature. Conidia were harvested by washing the slants with normal saline-0.0125% Tween 20 solution. The suspension was then filtered through a two-ply sterile gauze pad to remove any unwanted debris (hyphae and agar), resulting in a pure harvest of conidia. The cells were pelleted by centrifugation at 2,000 × g, washed once with normal saline, repelleted, and resuspended in 10 ml of normal saline. Cell counts were performed with a hemacytometer on each species, and volumes containing 10^6 and 10^7 cells were aliquoted and pelleted.
(ii) DNA isolation. Lysis buffer solutions used in both HSCD and PC procedures were optimized for each organism. C. albicans, S. cerevisiae, T. beigeli, A. fumigatus, and P. boydii pellets were resuspended in 500 μl of lysis buffer (LB) (0.05 M EDTA [pH 8.0], 0.3% sodium dodecyl sulfate [SDS]). C. neoformans was resuspended in 500 μl of LEM containing 0.5 M EDTA, 1 M Tris-HCl (pH 8), 1% Sarkosyl, and 0.2% proteinase K (19). F. solani was resuspended in 500 μl of LB containing 0.05 M EDTA, 0.1 M Tris-HCl (pH 8), and 0.5 NaCl (12). Samples were transferred to a homogenization tube (Bio 101, Inc., Vista, Calif.) containing beads and immediately processed in the FP 120 FastPrep cell disrupter (Bio 101, Inc.) for 30 s. All samples were processed twice, except for C. neoformans, which was processed four times. Between processing, the tubes were cooled on ice for 10 min. The samples were then centrifuged for 5 min at 14,000 × g to pellet cell debris.

(iii) DNA binding and elution. Supernatants were transferred to new tubes, and 300 μl of glass milk binding matrix (Bio 101, Inc.) was added. The supernatants and matrices were mixed by inversion for 2 to 3 min, followed by centrifugation for 2 min at 14,000 × g. Supernatants were discarded, and pellets were resuspended in 500 μl of 80% ethanol. The mixture was transferred to spin filters and centrifuged twice for 2 min at 14,000 × g to remove the last traces of ethanol. The spin filters containing the glass milk pellet were transferred to clean 250-μl glass tubes. The supernatants and matrices were mixed by inversion for 2 to 3 min, followed by centrifugation for 2 min at 20,000 × g. All samples were treated with RNase (RNase A; Sigma, St. Louis, Mo.) for 1 h at 37°C. A 1% agarose gel was run against the standard curve. To control for potential interday and interuser variation, the extractions of each organism were performed on at least three different days by two different individuals.

PC extraction. (i) Culture and harvesting of fungal strains. Culturing and harvesting of yeasts and filamentous fungi were performed as described above by a conventional PC extraction protocol (27).

(ii) Spheroplast formation. Yeast spheroplasts for C. albicans, S. cerevisiae, and T. beigeli were prepared by resuspending cells in 1.5 ml of spheroplast buffer (1 M sorbitol, 0.05 M sodium phosphate [monobasic], 0.1% 2-mercaptoethanol, 100 μg of Lyticase [Sigma] per ml) (7). Cells from C. neoformans and filamentous fungi were resuspended in 1 ml of spheroplast buffer plus 75 μl of Novozyme (20 mg/ml; Sigma) (19, 23). Pellets were resuspended by vortexing and then incubated for 45 min at 30°C. Upon completion, spheroplasts were pelleted by centrifugation for 10 min at 2,000 × g.

(iii) Cell lysis. Each pellet was resuspended in 800 μl (yeast) and 500 μl (filamentous fungi) of LB. To the filamentous fungi samples, an additional 62.5 μl of primer RP5 (107 CFU) was added. DNA was extracted overnight in a 70°C water bath for 10 min and then heated in a 70°C water bath for 10 min. The samples were then centrifuged for 5 min at 14,000 × g. The supernatant containing DNA was transferred to a homogenization tube (Bio 101, Inc., Vista, Calif.) containing beads and immediately processed in the FP 120 FastPrep cell disrupter (Bio 101, Inc.) for 30 s. All samples were processed twice, except for C. neoformans, which was processed four times. Between processing, the tubes were cooled on ice for 10 min. The samples were then centrifuged for 5 min at 14,000 × g to pellet cell debris.

(iv) DNA precipitation. Purified lysates were transferred to clean microcentrifuge tubes. To each tube, 32 μl of 5 M sodium chloride was added (followed by 2 volumes of 100% ethanol. DNA was precipitated for 1 h at −20°C and then pelleted at 4°C in a microcentrifuge at 14,000 × g for 30 min. The supernatant was decanted, and the pellets were rinsed with ice-cold 70% ethanol. Pellets were dried in a vacuum desiccator overnight. DNA was eluted in 10 μl of TE; DNA from 108 CFU was eluted in 100 μl of TE.

RFLP. Restriction digests were performed with 15 μl of DNA extracted by HSCD from 107 cells of C. albicans, C. neoformans, S. cerevisiae, T. beigeli, A. fumigatus, F. solani, P. boydii, and R. arrhizus. The DNA was digested with 10 U of EcoRI (Boehringer Mannheim, Indianapolis, Ind.) for 2 h at 37°C. The digests were analyzed on a 0.7% agarose gel.

PCR. DNA extracted by HSCD from 107 cells of C. albicans, C. neoformans, S. cerevisiae, T. beigeli, A. fumigatus, F. solani, P. boydii, and R. arrhizus was used for PCR. PCR samples were set up under standard reaction conditions (Qiagen, Santa Clarita, Calif.). For all organisms, oligonucleotides from 188 rRNA were used: FG-F, 5′-ATTTGAGGGGCGAAGTCGTTG-3′, and FG-R, 5′-CCCGATCTTAAGTAGCGATA-3′. PCR was performed as follows: 94°C for 30 s, 62°C for 1 min, and 72°C for 2 min, for 35 cycles with a final extension of 7 min. A 0.53-kb product was amplified (15). Each PCR product (10 μl) was analyzed on a 1% agarose gel.

RAPD. DNA extracted by HSCD from 107 cells of C. albicans, C. neoformans, S. cerevisiae, T. beigeli, A. fumigatus, F. solani, P. boydii, and R. arrhizus was used for RAPD analysis. The single oligonucleotide used was RPS: 5′-CCGCTAGCCTAGT-3′ (22). Amplification reactions were performed in 1.5 ml of MgCl2·50 mM KC1·10 mM Tris-HCl·0.01% (wt/vol) gelatin, which contained 20 mM (each) dATP, dCTP, dGTP, and dUTP (Boehringer Mannheim); 10 μl of primers (diluted 1:100); 2.5 U of Taq DNA polymerase (Boehringer Mannheim); 2.5 U of AmpliTag DNA polymerase Stoeckel fragment (Perkin-Elmer Cetus, Foster City, Calif.); 1 U of uracil DNA glycosylase (Boehringer Mannheim); and 1 μl (diluted 1:50) of DNA in 50 μl of PCR buffer. Samples were heated at 37°C for 15 min and then placed in a thermal cycler. The PCR was performed as follows: 95°C for 7 min, 95°C for 1 min, 35°C for 1 min, and 72°C for 2 min for 10 cycles and 95°C for 30 s, 45°C for 30 s, and 72°C for 1 min for 36 cycles followed by a 7-min extension period at 72°C. Each RAPD product (25 μl) was run on a 2% agarose gel.
multaneously is necessary (17). Moreover, the use of toxic chemicals such as PC further limits the use of conventional DNA extraction procedures in clinical microbiology laboratories (5, 6, 18, 25). While enzymatic methods are generally employed for obtaining consistent release of fungal DNA, the yields are often low (25, 26).

Recently, an HSCD system incorporating chaotropic reagents and lysing matrices for disruption of cell membranes was developed (FP 120 FastPrep). The cell disruption is caused by the collision between the fungal cell wall and the beads within the reaction tube. The effectiveness of the cell disruption process depends on the rate of collision and the energy of impact, which are functions of the speed settings (range, 4.0 to 6.5 m/s), and the specific gravity of the bead material used. The rate of collision is proportional to the speed, while the energy of impact is proportional to the square of the speed.

The HSCD system has been successfully applied to extractions of DNA and RNA from bacterial pathogens and in agricultural studies. For example, the FastPrep cell disrupter in combination with the FastRNA kit has been used for total RNA extraction from *Vibrio cholerae*, followed by reverse transcriptase PCR to detect viable cells of *V. cholerae* (1). RNA extracted from *Mycobacterium aurans*, *Streptococcus pyogenes*, and *Mycobacterium tuberculosis* by similar methods was found to be suitable for Northern blotting (9, 10, 13, 14). RNA extraction based on homogenization of plant tissue by high-speed reciprocal shaking in the presence of a mixture of sand and glass beads was successfully performed on plant samples (14). HSCD DNA extraction from agricultural soil was five times faster than previous methods and resulted in very pure DNA suitable for restriction digestion, cloning, and PCR amplification. The method provided far less shearing of DNA (predominantly 9 to 23 kb) than did previous bead methods.

![FIG. 1. PCR products from four yeasts and four filamentous fungi. Lane M, molecular size markers; lane (–), no organism.](http://jcm.asm.org)
used to extract DNA from sediments (0.5 to 9 kb) (3). DNA extracted from soybean plants by the HSCD system was also suitable for PCR (8). To our knowledge, HSCD heretofore has not been applied to medically important fungi for potential use in clinical microbiology laboratories.

The use of different lysis buffers bears note. For DNA isolation, conidia from filamentous fungi and blastoconidia from yeasts were pelleted and each pellet was resuspended in lysis solution from traditional extraction procedures. Our experience with different lysis solutions from traditional extraction procedures was confirmed in this system. Lysis solutions containing EDTA and SDS gave good yields of DNA for *C. albicans*, *S. cerevisiae*, *T. beigelii*, *A. fumigatus*, *P. boydii*, and *R. arrhizus*, but for *F. solani* and *C. neoformans*, different lysis solutions were necessary to obtain optimal yields within the same range. Additional lysis solutions could be explored for other fungal organisms to be used in the HSCD system.

DNA extraction by this rapid mechanical method was achieved within 1 h for up to 12 samples and was superior to PC extraction, which requires about 6 h. All samples were processed twice, except for those from *C. neoformans*, which, due to the tenacious capsule, were processed four times. A glass milk binding matrix was added to the supernatant, and following centrifugation, the pellet was resuspended and transferred to a spin column. The DNA was eluted from the column and run on a native agarose gel for confirmation of integrity. Up to the stage of binding and elution of the DNA, this procedure was able to be completed within 1 h.

In this study, DNA yields were not quantified by nucleic acid absorbance. Several recent studies have indicated that measurement of nucleic acid absorbance ratios at 260 nm/280 nm was inaccurate for determining the quantity or purity of nucleic acid preparations (16, 18, 24). Our findings confirm these observations. In order to further quantitatively assess the yields of DNA obtained from different lysis solutions from traditional extraction procedures. Our experience with different lysis solutions from traditional extraction procedures was confirmed in this system. Lysis solutions containing EDTA and SDS gave good yields of DNA for *C. albicans*, *S. cerevisiae*, *T. beigelii*, *A. fumigatus*, *P. boydii*, and *R. arrhizus*, but for *F. solani* and *C. neoformans*, different lysis solutions were necessary to obtain optimal yields within the same range. Additional lysis solutions could be explored for other fungal organisms to be used in the HSCD system.

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for PCR and RAPD analysis from 10^7 CFU. The quality and concentration of DNA were sufficient to obtain PCR products and distinguishable DNA patterns from RFLP and RAPD analysis. The rapidly extracted genomic DNA was of high molecular weight, and the integrity of DNA was equal to that obtained by a traditional PC extraction.

Further corroborating the effectiveness of DNA extraction by mechanical disruption of medically important filamentous fungi, van Burik et al. compared five nonenzymatic methodologies with an enzymatic method for greatest yield of high-quality DNA from A. fumigatus hyphae (26). The nonenzymatic methods were (i) glass bead pulverization with vortexing, (ii) grinding with mortar and pestle followed by glass bead pulverization with gentle rocking, (iii) use of 1% hydroxyacetethyltrimethylammonium bromide buffer followed by glass bead pulverization in a water bath sonicator, (iv) use of 1% hydroxyacetyltrimethylammonium bromide buffer followed by water bath sonication alone, and (v) grinding with mortar and pestle in liquid nitrogen, and the enzymatic method was Lyticase (Sigma) enzymatic fungal cell lysis. Genomic DNA yields were measured by visual reading of 2% agarose gels and spectrophotometry, with shearing assessed by the appearance on the gel. Fungal DNA yields were highest for the first method, followed by the fifth method; the other four methods yielded 10-fold-smaller amounts than did the first method. These results from A. fumigatus hyphae support our findings that high-speed vortexing with glass beads provides a rapid alternative for high-yield extraction of fungal DNA, especially from filamentous fungi.

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